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Ethylation and methylation of hemoglobin in smokers and non-smokers

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Two previous studies demonstrated elevated levels of 3-ethyladenine in smokers' urine, suggesting that cigarette smoke may contain a DNA ethylating agent. We hypothesized that such an agent would also lead to elevated levels of N-terminal *N*-ethylvaline in hemoglobin. N-terminal *N*-alkylated valines in hemoglobin can be measured using a modified Edman degradation, which employs pentafluorophenyl isothiocyanate to produce a pentafluorophenylthiohydantoin. The latter is quantified by gas chromatography-negative ion chemical ionization-mass spectrometry (GC-NICI-MS). We modified the published method to increase its sensitivity and selectivity, thereby allowing quantification of N-terminal *N*-ethylvaline. Modifications included the use of a deuterated peptide as the internal standard, the introduction of an HPLC purification step, and the use of tandem mass spectrometry (MS/MS) for detection and quantification of the analyte, 1-ethyl-5-isopropyl-3-pentafluorophenyl-2-thiohydantoin. We also quantified N-terminal *N*-methylvaline in the same samples. The mean level of N-terminal *N*-ethylvaline in the hemoglobin of smokers was 3.76 ± 2.77 pmol/g globin (n = 39), significantly higher than in non-smokers, 2.50 ± 1.65 pmol/g globin (n = 28), P = 0.023. The difference remained significant after correction for gender and age. The mean level of N-terminal *N*-methylvaline in smokers was 997 ± 203 pmol/g globin (n = 45) compared with 904 ± 149 pmol/g globin in non-smokers (n = 29); these values were not significantly different when corrected for gender and age. As levels of hemoglobin and DNA adducts often correlate, the results of this study support the proposal that cigarette smoke contains an as yet unidentified ethylating agent, which might be involved in DNA damage and tumor initiation.

Introduction

Cigarette smoking is responsible for ~30% of all cancer deaths in developed countries. In addition to lung cancer, cigarette smoking is an important cause of oral, oropharyngeal, hypopharyngeal, laryngeal and esophageal cancers as well as pancreatic cancer, bladder cancer and cancer of the renal pelvis (1,2). Other cancers related to cigarette smoking are cancers of the nose, stomach, renal body, liver, colon, cervix and myeloid leukemia (2–5).

Cigarette smoke carcinogens are the likely cause of cancer in smokers (6). Some such as *N*-nitrosamines and polycyclic aromatic hydrocarbons (PAH) require metabolic activation while others such as acetaldehyde and ethylene oxide do not. In either case, smokers are exposed to a constant flux of electrophiles which react with DNA to produce adducts. Considerable data indicate that these adducts cause permanent mutations in critical genes such as *ras* and *p53*, leading to loss of normal cellular growth control and ultimately to cancer (7,8). DNA adducts of tobacco-specific nitrosamines, PAH, aromatic amines, ethylene oxide and of unknown origin have been detected in tissues of smokers. However, technical problems have hindered these studies and relatively few DNA adducts have been convincingly characterized and quantified in smokers (G.P.Pfeifer *et al.*, submitted for publication).

Electrophiles, which react with DNA, also react with proteins such as hemoglobin and albumin. Hemoglobin adducts are potential surrogates for quantifying DNA damage by carcinogens (9,10). Advantages of hemoglobin adducts as dosimeters of carcinogen damage include the ease with which large quantities of hemoglobin are obtained, the relatively long lifetime of the red cell which permits accumulation of adducts, and the lack of any known repair mechanisms. Hemoglobin adducts of tobacco-specific nitrosamines, PAH, aromatic amines, ethylene oxide and other carcinogens have been quantified in smokers (10–17).

Two relatively recent studies demonstrated higher levels of 3-ethyladenine in the urine of smokers than in non-smokers (18,19). This could result from ethylation of adenine in DNA followed by depurination and excretion of 3-ethyladenine. The results of these studies suggest the presence in cigarette smoke of an unknown DNA ethylating agent, but there are no other data in the literature that support this potentially important proposal. Ethyl adducts in DNA, such as O^6-ethyldeoxyguanosine and O^4-ethylthymidine, have miscoding properties and are closely associated with cancer in animal studies (20–22). If smoking results in increased DNA ethylation, then increased hemoglobin ethylation also should be expected. Therefore, in this study, we analyzed hemoglobin of smokers and non-smokers for ethylated N-terminal valine. Methods for determining alkylated N-terminal valine of hemoglobin have been developed and applied by Tornqvist *et al.* (16,23,24), but there is only one report on ethylation. In that study, levels were below the detection limit of the method (24). We modified Tornqvist's method to increase its selectivity and sensitivity for detection of N-terminal *N*-ethylvaline. We also analyzed for N-terminal *N*-methylvaline in hemoglobin of smokers and non-smokers.

Abbreviations: GC-NICI-MS/MS, gas chromatography-negative ion chemical ionization-tandem mass spectrometry; GC-NICI-MS-SIM, gas chromatography-negative ion chemical ionization-mass spectrometry with selected ion monitoring; *N*-ethylvaline-PFPTH, 1-ethyl-5-isopropyl-3-pentafluorophenyl-2-thiohydantoin; *N*-methylvaline-PFPTH, 1-methyl-5-isopropyl-3-pentafluorophenyl-2-thiohydantoin; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; PAH, polycyclic aromatic hydrocarbons.

Materials and methods

Participants

Smokers were apparently healthy individuals participating in a study investigating the effects of reduction in smoking on exposure to tobacco smoke carcinogens. Each subject provided a 10 ml blood sample, collected in a tube containing EDTA. The samples used here were obtained at baseline, before smoking reduction. Samples from 45 smokers were randomly chosen for the analyses.

Non-smokers were participants in a study that assessed the effects of diet on N-terminal *N*-alkylvalines in hemoglobin (25). On two occasions, 1 month apart, each subject provided a 10 ml blood sample, collected in a tube containing EDTA. Samples from the first blood draw of 29 non-smokers were randomly chosen for the analyses. An additional nine samples were randomly chosen from the second blood sampling of these 29 subjects. The total number of non-smokers in the diet study was 114, and analysis of N-terminal *N*-ethylvaline and *N*-methylvaline in the hemoglobin of these subjects has been described (25). The method used in that study for N-terminal *N*-ethylvaline was different from the one described here, as it employed GC-NICI-MS-SIM, not GC-NICI-MS/MS. When the current improved method for N-terminal *N*-ethylvaline became available, all samples in the earlier study were re-analyzed by GC-NICI-MS/MS, without any effect on the conclusions. A subset of the 114 non-smoker samples, the ones described here, were analyzed for N-terminal *N*-ethylvaline at the same time and by the same method as the smokers' samples described here. Therefore, only this subset was used in the present comparison. (The worked up fractions of the remaining non-smoker samples had been frozen for an additional 6 months prior to GC-NICI-MS/MS analysis for *N*-ethylvaline-PFPTH and the results were therefore not included in the present comparison with smokers. However, inclusion of those results did not change the conclusions of the present study.) All N-terminal *N*-methylvaline analyses were by the same method, GC-NICI-MS-SIM. In this paper, we only present data for the same non-smokers for whom we report measurements of N-terminal *N*-ethylvaline. Inclusion of all data did not change the conclusions reported here.

Both studies were approved by the University of Minnesota Research Subjects' Protection Programs Institutional Review Board Human Subjects Committee.

Chemicals

Formamide (USB, Cleveland, OH), hexane (Fisher Scientific, Pittsburgh, PA), dodecane (Fisher Scientific), pentafluorophenyl isothiocyanate (Fluka, Ronkonkoma, NY) [D_3]*N*-methylvaline-HLTPF (SynPep, Dublin, CA), and *N*-methylvaline (Sigma Chemical Co., St Louis, MO) were purchased. *N*-Ethylvaline, 1-ethyl-5-isopropyl-3-pentafluorophenyl-2-thiohydantoin (*N*-ethylvaline-PFPTH), 1-methyl-5-isopropyl-3-pentafluorophenyl-2-thiohydantoin (*N*-methylvaline-PFPTH), [D_3]*N*-methylvaline, [D_3]*N*-methylvaline-PFPTH, and 1-methyl-5-isopropyl-3-phenyl-2-thiohydantoin were synthesized essentially as described by Rydberg *et al.* (26).

Analysis of N-terminal N-ethylvaline and N-methylvaline in globin

Red cells were separated from serum immediately after collection by centrifugation at 1000 *g* for 15 min at 4°C. The red cells were washed twice with 20 ml saline (0.9% in H_2O) and stored at –20°C until analysis. For isolation of globin, 2 ml of red cells were lysed with 3 ml H_2O in 30 ml Nalgene centrifuge tubes (Nalge Co., Rochester, NY) and 23 ml 0.5% HCl in cold isopropanol was added, and the mixture was agitated. The mixture was centrifuged at 3000 *g* for 20 min at 4°C. The acidified hemoglobin supernatant was transferred into a 50 ml centrifuge tube and globin was precipitated by slow addition, with mixing, of 20 ml cold ethyl acetate. The mixture was allowed to stand in ice for 20 min, then centrifuged to separate the precipitate. The precipitate was washed twice with ethyl acetate and once with pentane, then dried under a stream of N_2. Globin was stored at –20°C until analysis. Just prior to analysis, the globin was dried *in vacuo* for 16 h.

Globin (20 mg) was dissolved in 1.5 ml formamide in a 5 ml glass centrifuge tube. The formamide was purified before use by extraction with two equal volumes of pentane (16). The globin samples were vortexed for 1 h for complete dissolution. The samples were then neutralized by addition of about 25 µl 1 N NaOH. The peptide [D_3]*N*-methylvaline-HLTPF (5 ng, 6.85 pmol) was added to each sample as internal standard. The internal standard was initially dissolved in DMSO, then diluted in formamide. After addition of the internal standard, 7 µl of pentafluorophenyl isothiocyanate was added and the samples were shaken overnight at room temperature. The samples were then heated at 45°C for 90 min to complete the reaction. The pH was adjusted to 6.5–7.0 with 10–15 µl 1 N NaOH. (During the derivatization reaction, the pH drops by ~1 U, which favors splitting of the derivative from the globin. Readjusting the pH to 7 minimizes the formation of emulsions during subsequent extractions.) The samples were extracted three times with 2.5 ml of ether. The extracts were combined into a 5 µl centrifuge tube and concentrated to dryness under a stream of N_2. The residue was re-dissolved in 1 ml hexane and washed twice with 2.5 ml H_2O, twice with 3 µl freshly prepared 0.1 M sodium carbonate, and again twice with H_2O. The hexane layers were centrifuged for 10 min and all H_2O was removed. Dodecane (5 µl) was added, the tubes were placed in a beaker of warm H_2O, and the hexane was removed under a gentle stream of N_2. The tubes were removed at the point of near dryness. The extracts were then transferred to 200 µl HPLC autosampler microvials with 3×65 µl 95% hexanes/5% isopropanol. The samples were stored at –20°C until HPLC purification.

A normal phase Pinnacle 5 mm cyano HPLC column (4.6×250 mm, Restek, Bellefonte, PA) with a cyano guard cartridge (3.0×4 mm i.d., Phenomenex, Torrance, CA) was used for further purification of the samples. Detection was by UV at 254 nm. Solvent A was 100% hexane and solvent B was 95% hexane:5% isopropanol. The flow rate was 1 ml/min.

1-Methyl-5-isopropyl-3-phenyl-2-thiohydantoin (0.16 mg) was injected to calibrate the system and determine the appropriate collection window. It eluted at 15.6 min. The solvent program was 100% A for 10 min, then from 100% A to 100% B in 20 min, then return to 100% A. Eluant was collected from 6–14.5 min, which included *N*-ethylvaline-PFPTH (retention time, 7.6 min), *N*-methylvaline-PFPTH (9.6 min), and [D_3]*N*-methylvaline-PFPTH (9.8 min).

Dodecane (10 µl) was added to each 15 ml tube containing the collected HPLC fraction. The hexane was removed under a gentle stream of N_2. The residue was transferred into a 200 µl microvial with 3×65 µl portions of hexane. The hexane was carefully evaporated under a gentle stream of N_2. The samples, now in dodecane, were stored at –20°C until further analysis.

Gas chromatography-negative ion chemical ionization-tandem mass spectrometry (GC-NICI-MS/MS) was carried out on a Finnigan TSQ 7000 instrument (Thermoquest, San Jose, CA) interfaced with a CTC A200SE autosampler (Leap Technologies, Carrboro, NC) and a HP5890 series II GC (Agilent Technologies, Palo Alto, CA). The GC was fitted with a 30 m×0.32 mm ×0.25 μm film thickness DB-1301 column (Agilent Technologies) and a 2 m×530 μm uncoated deactivated fused silica precolumn. The carrier gas was He, at 1.4 ml/min constant flow. Two μl of each sample were injected via splitless injection with a gooseneck splitless liner. The splitless vent flow was 120 ml/min. The purge open time was 0.75 min. The injector temperature was 225°C. The MS interface temperature was 250°C. The GC oven was programmed as follows: 100°C for 2 min, then 10°C/min until 140°C, then 2°C/min until 165°C, then hold for 15 min, then 15°C/min until 250°C, then hold for 5 min.

CI source parameters were as follows: filament emission current, 300 μA; electron energy, 200 eV; ion source temperature, 150°C. Methane was the reagent gas. For the GC-NICI-MS/MS determination of *N*-ethylvaline-PFPTH, *m/z* 351 was selected, and scanned at 0.5 amu resolution, scan time 1.0 s. The collision gas was Ar, collision energy 20 eV. The transition *m/z* 351→322 [(M-1)-C_2H_5], which was the largest daughter ion, was monitored. For [D_3]*N*-methylvaline-PFPTH, the transition *m/z* 340→206 was monitored.

For analysis of *N*-methylvaline-PFPTH by gas chromatography-negative ion chemical ionization-mass spectrometry with selected ion monitoring (GC-NICI-MS-SIM), the reagent gas was methane. CI source parameters were as follows: filament emission current, 700 μA; electron energy, 150 eV, ion source temperature, 150°C. For *N*-methylvaline-PFPTH, *m/z* 337 (M-1) was monitored and for [D_3]*N*-methylvaline-PFPTH, *m/z* 340 was monitored. The peak width was 0.5 amu and the scan time was 0.3 s.

Retention times were as follows: *N*-ethylvaline-PFPTH, 19.68 min; *N*-methylvaline-PFPTH, 19.37 min, [D_3]*N*-methylvaline-PFPTH, 19.35 min. Standard curves for *N*-ethylvaline-PFPTH and [D_3]*N*-methylvaline-PFPTH were generated by GC-NICI-MS/MS. Levels of *N*-ethylvaline-PFPTH in each sample were corrected for loss of deuterated internal standard. Levels of *N*-methylvaline-PFPTH were similarly calculated using GC-NICI-MS-SIM.

Statistical analysis

For nine of the non-smokers, analyses were carried out on two blood draws 1 month apart. When two values were available, these were averaged and the resulting mean values included with the rest of the non-smokers. When one of the two samples was non-quantifiable, the remaining value was used. For samples in which *N*-ethylvaline-PFPTH was not detected, we used half the detection limit. Levels of N-terminal *N*-ethylvaline and *N*-methylvaline in smokers versus non-smokers were compared using the two-sided two-sample *t*-test and the two-sided Wilcoxon rank-sum test. Levels of N-terminal *N*-methylvaline in non-smokers on two occasions were compared using a paired *t*-test. Age distributions of smokers and non-smokers were compared using the two-sided two-sample *t*-test. Gender distribution of smokers and non-smokers was compared using the chi-squared test. Effects of age and gender on adduct level differences in smokers and non-smokers were compared by multiple regression. Correlations of N-terminal *N*-methylvaline and *N*-ethylvaline with other parameters were evaluated using Excel regression programs.

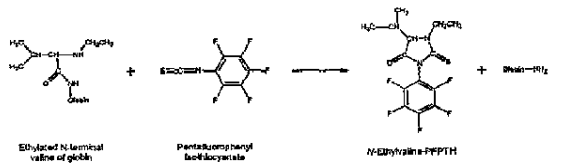

Fig. 1. Formation of *N*-ethylvaline-PFPTH in the modified Edman degradation of N-terminal N-ethylated globin.

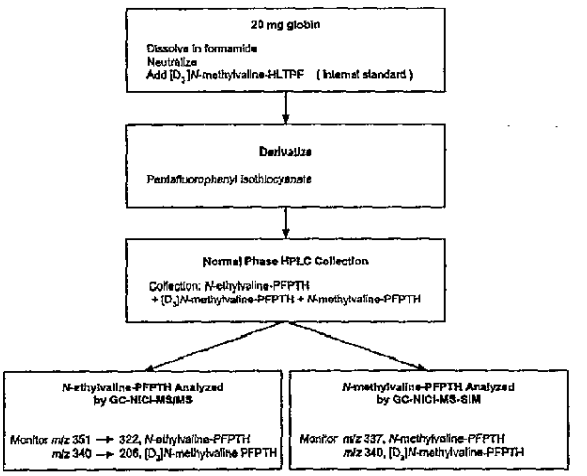

Fig. 2. Scheme for analysis of *N*-ethylvaline-PFPTH and *N*-methylvaline-PFPTH as formed in the modified Edman degradation of globin.

Results

The method used here is based on the modified Edman degradation developed by Tornqvist *et al.* (Figure 1) (16,23). Globin with an *N*-alkylated N-terminal valine is allowed to react with pentafluorophenyl isothiocyanate. This results in cleavage from the protein of 1-alkyl-5-isopropyl-3-pentafluorophenyl-2-thiohydantoin (*N*-alkyl-PFPTH). In the present study, the alkyl group is methyl or ethyl. Extensive studies by Tornqvist *et al.* (16,23) have validated this methodology for various alkyl groups, most notably hydroxyethyl, resulting from reaction of ethylene oxide with globin. However, the sensitivity of the published method was not adequate for determination of N-terminal *N*-ethylvaline in globin at the levels expected in our study. Therefore, we modified the method in several ways.

Our method for quantification of N-terminal *N*-ethylvaline in globin is summarized in Figure 2. [D_3]*N*-Methylvaline-HLTPF was used as internal standard. The four amino acids to which the deuterated *N*-methylvaline is attached are the same as those at the N-terminal end of the β-chain of human hemoglobin. The fifth amino acid, F, was chosen to provide a chromophore to conveniently allow HPLC purity checks of the peptide. In some experiments, we also used the corresponding deuterated *N*-ethylvaline peptide, but subsequent studies showed that this was contaminated with small amounts of the undeuterated *N*-ethylvaline peptide, which substantially interfered with our analysis. In our hands, [D_3]*N*-methylvaline-HLTPF was a superior internal standard to [D_5]ethylated globin because its concentration could be more accurately determined. [D_3]*N*-Methylvaline-HLTPF was added to the globin solution

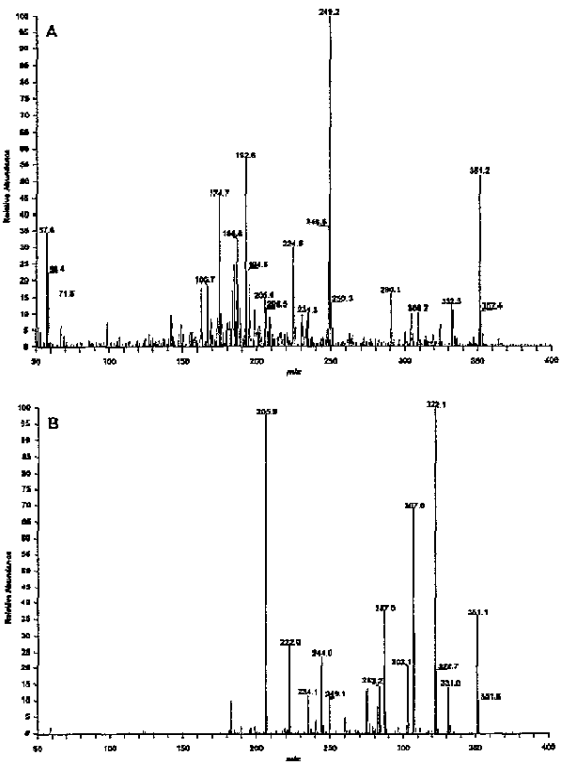

Fig. 3. (A) Full scan NICI-MS of *N*-ethylvaline-PFPTH and (B) MS/MS of *m/z* 351.

and derivatization was carried out with pentafluorophenyl isothiocyanate.

In the next step, we used normal phase HPLC to further purify the sample. Initial attempts to quantify *N*-ethylvaline-PFPTH using solvent partitioning clean up steps proved to be insufficient for removal of interfering substances. We also experimented with a reverse phase HPLC step, but removal of the collected methanol–H_2O eluting solvent by centrifugal evaporation under reduced pressure led to large losses of analyte. In the normal phase HPLC system, hexane was employed as eluting solvent, and this could be gently removed under a stream of N_2 without loss of analyte. During the solvent removal step, we added the higher boiling solvent dodecane to prevent complete evaporation of solvent, thus helping to prevent analyte loss. The dodecane also prevented evaporative losses during storage prior to GC-NICI-MS/MS analysis. *N*-Methylvaline phenylthiohydantoin was used as a retention time marker in the normal phase HPLC step, and the region including *N*-ethylvaline-PFPTH, *N*-methylvaline-PFPTH, and [D_3]*N*-methylvaline-PFPTH was collected. Blank injections demonstrated that there was no carryover of analyte between HPLC runs.

A CI-MS of *N*-ethylvaline-PFPTH is shown in Figure 3A. For the GC-NICI-MS/MS analysis, we selected *m/z* 351 (M-H). The daughter ion spectrum of *m/z* 351 is illustrated in Figure 3B. We selected the transition *m/z* 351→322 (M-H-C_2H_5) for the MS/MS analysis. GC-NICI-MS/MS analysis provided considerably greater sensitivity and cleaner chromatograms than were achieved by GC-NICI-MS-SIM analysis of *N*-ethylvaline-PFPTH, *m/z* 351. The internal standard produced

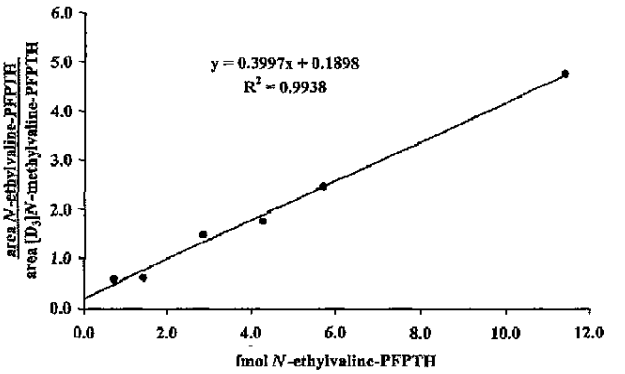

Fig. 4. Calibration curve for GC-NICI-MS/MS analysis of *N*-ethylvaline-PFPTH and [D_3]*N*-methylvaline-PFPTH internal standard, R^2 = 0.994.

[D_3]*N*-methylvaline-PFPTH, which was monitored at *m/z* 340→206. For analysis of *N*-methylvaline-PFPTH, which was present in greater quantities, GC-NICI-MS-SIM, *m/z* 337 (M-H), was satisfactory. The internal standard was monitored at *m/z* 340.

Calibration curves were constructed prior to each analysis. A calibration curve for *N*-ethylvaline-PFPTH is illustrated in Figure 4. Typical chromatograms of *N*-ethylvaline-PFPTH and *N*-methylvaline-PFPTH obtained upon analysis of globin from smokers and non-smokers are shown in Figure 5A–D.

Assay precision was determined by dividing a smoker's globin sample into four aliquots and analyzing each. The results were 6.26 ± 0.80 pmol *N*-ethylvaline/g globin (CV = 12.7%). Recoveries of [D_3]*N*-methylvaline-HLTPF were generally 10–20%. The instrumental detection limit for *N*-ethylvaline-PFPTH by GC-NICI-MS/MS was 0.7 fmol on column. The detection limit in globin samples was ~0.4 pmol/g globin.

In some samples, co-eluting peaks prevented quantification of *N*-ethylvaline-PFPTH. This occurred in four of 45 smoker samples and in six of 38 non-smoker samples (from 29 non-smokers), and these samples were excluded from the analysis. In the non-smoker samples, five of the six instances occurred among the nine individuals from whom we drew blood on two occasions, and consequently we used the remaining value. Thus, we had 39 smoker data points and 28 non-smoker data points for *N*-ethylvaline-PFPTH. There were no co-eluting peaks, which prevented quantification of *N*-methylvaline PFPTH.

Among the non-smokers, data were available for N-terminal *N*-methylvaline in hemoglobin of nine subjects obtained on two occasions 1 month apart. Levels were (mean ± SD) 808 ± 172 pmol/g globin on the first occasion and 893 ± 212 pmol/g globin on the second. Analysis of the data demonstrated that there was no significant difference in individual values on the two occasions (P = 0.27). For N-terminal *N*-ethylvaline, only four values were available on both occasions, as mentioned above. Among these four pairs of values, there were three below the detection limit (two from one individual). Therefore, intraindividual variation in the non-smoker N-terminal *N*-ethylvaline data could not be analyzed.

Smokers ranged in age from 29 to 66 (mean ± SD, 47.5 ± 8.4) and were 50% male. They smoked from 16 to 38 cigarettes per day (mean 24.5 ± 5.4). Non-smokers ranged in age from 20 to 57 (mean 29.4 ± 9.2) and were 34%

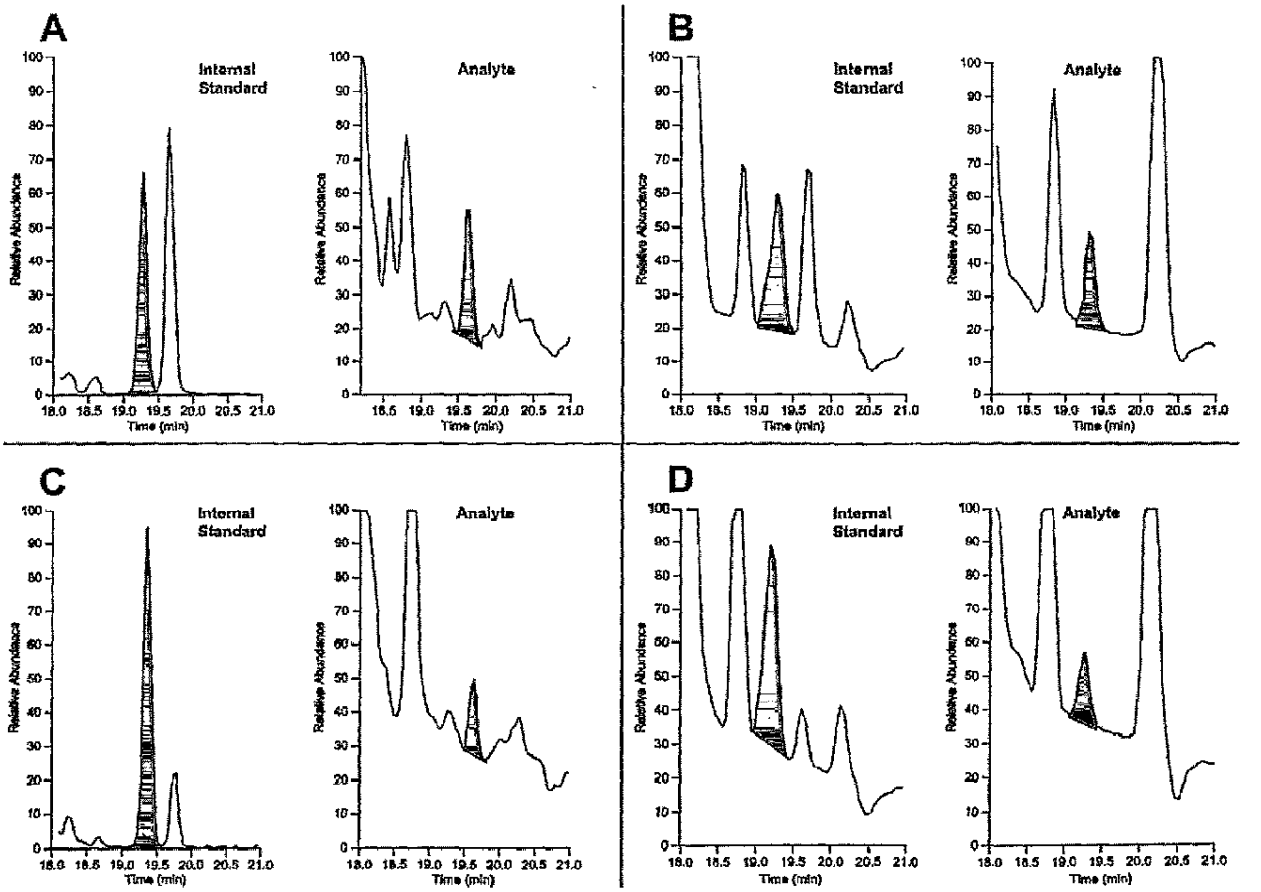

Fig. 5. (A) GC-NICI-MS/MS chromatogram of *N*-ethylvaline-PFPTH released from N-terminal *N*-ethylvaline in hemoglobin from a smoker. Left panel, *m/z* 340→206 of [D_3]*N*-methylvaline-PFPTH released from internal standard [D_3]*N*-methylvaline-HLTPF; right panel, *m/z* 351→322 of *N*-ethylvaline-PFPTH. (B) GC-NICI-MS-SIM chromatogram of *N*-methylvaline-PFPTH released from N-terminal *N*-methylvaline in hemoglobin from a smoker. Left panel, *m/z* 340 from internal standard [D_3]*N*-methylvaline-PFPTH; right panel, *m/z* 337 from *N*-methylvaline-PFPTH. Shaded peaks are the analytes of interest. Results of analyses of hemoglobin from non-smokers for N-terminal *N*-ethylvaline and N-terminal *N*-methylvaline are shown in (C) and (D), respectively.

male. Smokers were significantly older than non-smokers ($P < 0.001$). There was no significant difference in gender among the smokers and non-smokers. Levels of N-terminal *N*-ethylvaline in smokers and non-smokers are summarized in Figure 6. The mean level in smokers, 3.76 ± 2.77 pmol/g globin ($n = 39$) was significantly higher than in non-smokers, 2.50 ± 1.65 ($n = 28$), $P = 0.023$ (two-sided *t*-test) and $P = 0.025$ (Wilcoxon two-sided test). The difference was still significant after correction for gender and age ($P = 0.019$). Levels of N-terminal *N*-methylvaline in smokers and non-smokers are summarized in Figure 7. The mean level in smokers, 997 ± 203 pmol/g globin ($n = 45$), was slightly higher than in non-smokers, 904 ± 149 pmol/g globin ($n = 29$). This difference was of borderline significance, $P = 0.037$ (two-sided *t*-test) and $P = 0.071$ (Wilcoxon two-sided test). When corrected for gender and age, the difference between smokers and non-smokers was not significant ($P = 0.21$).

Among the smokers, there was no significant statistical association of N-terminal *N*-ethylvaline or N-terminal *N*-methylvaline levels with age, gender, number of cigarettes smoked per day, alcohol consumption, levels of urinary cotinine or levels of urinary total 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol. Among the non-smokers, there was no significant statistical association of levels of either hemoglobin adduct with age or gender. Levels of N-terminal *N*-ethylvaline and N-terminal *N*-methylvaline did not correlate with each other in either smokers or non-smokers.

Discussion

The results of this study support the proposal that cigarette smoke contains an ethylating agent. Our data demonstrate that levels of N-terminal *N*-ethylvaline in the globin of smokers are ~50% higher than in non-smokers, a statistically significant difference. Two reports showed increased levels of 3-ethyladenine in smokers' urine. In one study, the amount of urinary 3-ethyladenine was more than five times greater on days when individuals smoked than on days when they did not (18). Urinary 3-ethyladenine was also five to eight times higher in smokers than in non-smokers in that investigation. In a second study, urinary 3-ethyladenine increased in some smokers, on days when they smoked, and a correlation was seen between cigarettes per day and 3-ethyladenine excretion (19). 3-Ethyl-

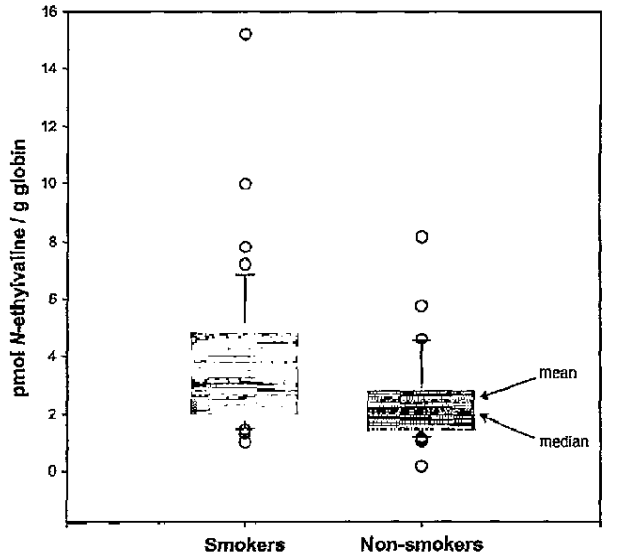

Fig. 6. Box plots of N-terminal *N*-ethylvaline in smokers and non-smokers. Boxes represent the 25th–75th percentile, outer lines represent the 10th–90th percentile, and dots represent outliers.

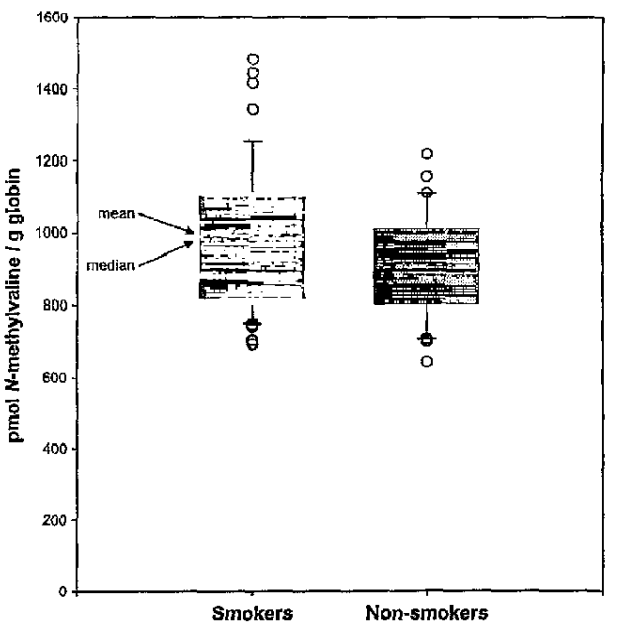

Fig. 7. Box plots of N-terminal *N*-methylvaline in smokers and non-smokers. See Figure 6 legend.

adenine in urine most likely results from reaction of an ethylating agent with adenine in nucleic acids followed by depurination or repair. Murthy *et al.* (27) have demonstrated a linear relationship between ethylation of DNA and hemoglobin in mice treated with ethyl methanesulfonate. Recently, Godschalk *et al.* (28), in an abstract, reported higher levels of O^4-ethylthymidine in lung DNA of smokers than in non-smokers. The collective data in smokers, although carried out in different laboratories with different protocols, indicate that nucleic acids and hemoglobin are being ethylated. Our data also indicate that hemoglobin is ethylated in non-smokers.

There are several potential sources of an ethylating agent in cigarette smoke. One is nitrosation of ethylamine. Mainstream smoke contains relatively high levels of nitrogen oxides, 100–600 μg/cigarette (29). The nitrosating potential of cigarette smoke has been demonstrated by studies, which found higher levels of nitrosamino acids in the urine of smokers compared with non-smokers (30–32). Smoke also contains ~1 μg ethylamine/cigarette (33). Nitrosation of ethylamine would produce the ethyl diazonium ion, a well-known ethylating agent. Ethyl diazonium ion is also formed upon hydrolysis of *N*-nitroso-*N*-ethylurea or metabolic α-hydroxylation of *N*-nitrosodiethylamine or *N*-nitrosoethylmethylamine (34). These possibilities seem less likely because *N*-nitroso-*N*-ethylurea is not known to be present in cigarette smoke and *N*-nitrosodiethylamine and *N*-nitrosoethylmethylamine occur in relatively low concentrations, 3 and 13 ng/cigarette, respectively (29). Nitrosation of ethanol could produce ethyl nitrite, another potential nitrosating agent. There are presently no reports of ethyl nitrite in cigarette smoke. Another possible source is acetaldehyde, which is found in smoke at levels of ~1 mg/cigarette, and is the primary metabolite of ethanol (35). Acetaldehyde could react with N-terminal valine to produce a Schiff base, which would then be required to undergo endogenous reduction to give N-terminal *N*-ethylvaline. Some support for this proposal can be found in the studies of Tornqvist *et al.* (36) who noted increased levels of N-terminal *N*-methylvaline in mice treated with formaldehyde. It is not clear how reduction would occur. It also appears unlikely that acetaldehyde could give rise to 3-ethyladenine, although there are reports that N^2-ethyldeoxyguanosine is elevated in individuals who consumed ethanol (37). There was no correlation between alcohol consumption and N-terminal *N*-ethylvaline levels in the present study.

Levels of N-terminal *N*-methylvaline were ~10% higher in smokers than in non-smokers, a difference of borderline statistical significance, which disappeared when corrected for age and gender. Tornqvist *et al.* (36) found 540 pmol/g Hb *N*-methylvaline in smokers compared to 500 pmol/g Hb in non-smokers in a small study. A second investigation by this group examined N-terminal *N*-methylvaline levels in a group of monozygotic twins discordant for smoking (38). They found 24% higher levels in smokers, a significant difference. Bader *et al.* (39) found higher levels of N-terminal *N*-methylvaline in smokers than in non-smokers. Overall, there may be some increase in N-terminal *N*-methylvaline in smokers compared with non-smokers. There is clearly a high endogenous level of N-terminal *N*-methylvaline in globin of humans and other species. This can also be seen in the present results, where levels of N-terminal *N*-methylvaline were ~250–350 times greater than those of N-terminal *N*-ethylvaline. Any effect of smoking would be seen only above the endogenous levels. Tornqvist *et al.* (36,38) argue that *S*-adenosyl methionine is the major source of endogenous N-terminal *N*-methylvaline. Other potential sources are methyl chloride, *N*-nitrosodimethylamine, and formaldehyde but the contribution of these agents appears to be less probable (36). Enzymatic methylation of proteins is well established but there are no reports on methylation of N-terminal valine of hemoglobin (40,41). Methylating nitrosamines such as the tobacco-specific nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and *N*-nitrosodimethylamine would be two likely

Table I. Representative levels of hemoglobin adducts in smokers and non-smokers

Analyte	Representative levels (fmol/g)			
	Source of adduct	Smokers	Non-smokers	Reference
4-Hydroxy-1-(3-pyridyl)-1-butanone	NNK, *N'*-nitrosonornicotine	55	27	(11,12)
4-Aminobiphenyl	4-Aminobiphenyl	1050	300	(10)
3-Aminobiphenyl	3-Aminobiphenyl	18	8	(12)
Benzo[*a*]pyrene-7,8,9,10-tetraol	Benzo[*a*]pyrene	105	68	(50)
o-Toluidine	*o*-Toluidine	2700	2200	(12)
N-Ethylvaline	?	3760	2500	This study
N-(2-Carbamoylethyl)valine	Acrylamide	116 000	31 000	(13)
N-(2-Cyanoethyl)valine	Acrylonitrile	252 000	4900	(14)
N-(2-Hydroxyethyl)valine	Ethylene oxide	242 000	12 900	(14)
N-Methylvaline	?	997 000	904 000	This study

sources of additional hemoglobin methylation in smokers (42). Measurements of 3-methyladenine in urine are complicated by its presence in diet and by background levels. Nevertheless, two controlled studies demonstrated increased excretion of 3-methyladenine in the urine of smokers (18,19). Mixed results have been obtained in studies of 7-methylguanine in DNA from smokers and non-smokers (43).

Representative data for some carcinogen hemoglobin adducts which have been quantified in smokers are summarized in Table I. In most cases, higher adduct levels are found in smokers than in non-smokers. Adduct levels range from 18 fmol/g globin (3-aminobiphenyl) to 997 000 fmol/g globin (*N*-methylvaline) in smokers. The carcinogens which produce relatively lower adduct levels such as NNK, *N'*-nitrosonornicotine, 3- and 4-aminobiphenyl, and benzo[*a*]pyrene are generally present in tens or hundreds of nanograms per cigarette, and all require metabolic activation prior to binding (44). Carcinogens which produce higher adduct levels such as acrylonitrile and ethylene oxide are present in microgram amounts per cigarette and do not require metabolic activation. The compounds producing lower hemoglobin adduct levels are generally stronger carcinogens in laboratory animals than those producing higher adduct levels, suggesting that reactions with protein may be protective, perhaps by competing with DNA reactions. Levels of *N*-ethylvaline are intermediate between these two groups. However, there are also substantial amounts of *N*-ethylvaline adducts in non-smokers indicating the presence of a relatively abundant non-tobacco related precursor.

Correlations of hemoglobin adduct levels with cigarettes smoked per day or urinary cotinine have been observed in some studies. Levels of 4-aminobiphenyl hemoglobin adducts correlated with number of cigarettes/day up to 20 but not from 20 to 90 cigarettes/day (10). No correlation of 4-aminobiphenyl-hemoglobin adducts, 4-hydroxy-1-(3-pyridyl)-1-butanone releasing hemoglobin adducts, or benzo[*a*]pyrene hemoglobin adducts with urinary cotinine was observed (12,45,46). Levels of hemoglobin adducts of ethylene oxide, acrylamide, and acrylonitrile did correlate with cigarettes per day (13,14,47,48). Mixed results have been obtained in studies of the relationship of N-terminal *N*-methylvaline to cigarettes per day (36,38,49). It appears that the strongest correlations between hemoglobin adduct levels and cigarettes per day or urinary cotinine are observed when the substrates (e.g. ethylene oxide, acrylamide, acrylonitrile) require no metabolic activation. This is reasonable since interindividual variation in metabolism of carcinogens such as 4-aminobiphenyl, benzo[*a*]pyrene, and tobacco-specific nitrosamines could significantly impact adduct levels. Based on these considerations, one could speculate that the precursor to N-terminal *N*-ethylvaline requires metabolic activation.

A limitation of this study was that the smokers and non-smokers were selected from different groups. The smoker samples were selected from a study investigating the effects of reduction in smoking on carcinogen uptake. The non-smoker samples were from a study investigating the effects of diet on hemoglobin adduct formation. A better design would have been to recruit matched groups of smokers and non-smokers. Nevertheless, N-terminal *N*-ethylvaline levels were still higher in smokers than in non-smokers, when corrected for differences in gender and age. Larger studies designed specifically to address this question will be required to confirm our results.

In summary, the results of this study demonstrate that ethylation of N-terminal valine of hemoglobin is ~50% higher in smokers than in non-smokers, a significant difference. These results are consistent with previous studies, which demonstrate increased excretion of 3-ethyladenine in the urine of smokers. The results indicate the presence in cigarette smoke of an as yet unidentified ethylating agent, which could contribute to the carcinogenic properties of smoke.

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